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Isolation of rapeseed genes expressed early and specifically during development of the male gametophyte

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stract

cDNA subtraction and differential hybridization strategy was used to isolate cDNAs expressed early during ale gametophyte development in the important crop species *Brassica napus*. Three cDNAs, corresponding to nes highly and specifically expressed at the tetrad and microspore stages, are presented here. The analysis of e of them, named *BnM3.4*, by *in situ* hybridization, showed that it is expressed specifically and at a high level in e rapeseed microspore. The specificity in its profile of expression is most likely transcriptionally controlled as a milar pattern of expression was also observed in *Arabidopsis thaliana* plants transformed by the *BnM3.4* promoter ked to the reporter GUS-coding sequence. The putative *BnM3.4* promoter contains three dispersed copies of a tif described previously in the promoters of several genes expressed in the male gametophyte. The *BnM3.4* gene codes a predicted novel proline-rich protein of 23.4 kDa which may interact with cytoskeletal components or ve a structural role in the cell wall.

troduction

llen development in angiosperm plants occurs in a ecialized floral organ, the anther. It starts by a se- s of mitotic divisions of archeosporial cells from the mary sporogenous layer leading to the formation of eiocytes. Each meiocyte undergoes meiosis to gen- ate a tetrad of four haploid microspores surrounded a callose wall, whose digestion then liberates them o the anther locule. A complex extracellular ma- x composed of intine and exine is built around the ale gametophyte after microspore differentiation. An ymmetric cell division of each microspore produces e bicellular pollen grain in which the large vegetative ll encloses the smaller generative cell. This latter ll has a condensed nucleus and a reduced cytoplasm. the majority of plant species, the second mitotic vision of the generative cell occurs after pollination within the growing pollen tube producing two sperm cells. In some genera such as *Brassica* this second pollen mitosis occurs earlier during pollen maturation. The pollen grain is hence released from the anther locule in a tricellular form. This developmental programme is necessary to prepare the pollen grain for efficient interaction with the stigma, for rapid germination and pollen tube growth, and for successful delivery of sperm cells to the ovules (Bedinger *et al.*, 1994).

Despite the wealth of descriptive studies on the structural and physiological aspects of pollen formation, our understanding of the molecular events remains rather limited. Our main goal is to extend our knowledge on microspore differentiation and development through the identification and characterization of novel genes specifically expressed during early stages of microgametogenesis. Such genes may also provide alternative methods for controlling male fertility in economically important plants, such as in our case *Brassica napus*. To identify genes that might be

The nucleotide sequence data reported will appear in the MBL, GenBank and DDBJ Nucleotide Sequence Databases under he accession number AF136223 (*BnM3.4*).

implicated in the developmental process, differential screening of cDNA libraries constructed from whole anthers has been the most commonly used approach. A number of genes have been shown to be expressed in developing pollen, as well as in the tapetum or other sporophytic tissues of the anther (Ursin *et al.*, 1989; Koltunow *et al.*, 1990; Nacken *et al.*, 1991; Theerakulpisut *et al.*, 1991; Paul *et al.*, 1992; Aguirre and Smith, 1993; Robert *et al.*, 1993; Bucciaglia and Smith, 1994; Ross and Murphy, 1996). Some pollen-specific genes expressed after the first mitotic division and presumably playing a role in pollen development, maturation, germination or pollen tube growth have also been described (Hanson *et al.*, 1989; Brown and Crouch, 1990; Shen and Hsu, 1992; Weterings *et al.*, 1992; Brander and Kuhlemeier, 1995; Stanchev *et al.*, 1996; Yu *et al.*, 1998). Finally, so far, a single tobacco gene has been identified that shows a microspore-specific expression (Oldenhof *et al.*, 1996),

We report the isolation of cDNAs from *B. napus* that are expressed during early pollen development. During this work, we developed a novel combined approach by differential screening of a microspore cDNA library with a subtracted probe enriched for microspore-specific sequences. We describe here in detail the characterization of the structure and expression of the *BnM3.4* gene corresponding to the microspore-specific cDNA M3.

Materials and methods

Plant material

Plants of *B. napus* L. cv. Brutor were grown in an open field or under standard greenhouse conditions and were used for RNA and DNA isolation. *Arabidopsis thaliana* (Wassilevskija ecotype) plants used for transformation (Bechtold *et al.*, 1993) were grown in a greenhouse under standard conditions. Transgenic *A. thaliana* T_1 seedlings were selected in a greenhouse on sand, sub-irrigated with water containing Basta herbicide (7.5 mg/l phosphinothricine). Two months later, T_2 seeds were harvested individually. *In vitro* culture of seedlings for segregation analysis was done in a culture chamber on *A. thaliana* medium (Estelle and Sommerville, 1987) containing 5 mg/l of phosphinothricine as selective agent.

RNA isolation and poly(A)$^+$ RNA purification

Several grams of rapeseed flower buds from 0.2 mm to more than 4 mm in length were harvested in 4 different classes according to bud length. Bud length was measured from the base to the tip of the outermost sepal. Male gametophytes from graded floral buds were isolated and purified as previously described (Albani *et al.*, 1990) with a few modifications. After disruption of the buds in a blender with a solution of 10% sucrose (pH 7), the resulting suspension was filtered through 80 μm nylon mesh. Only the suspension containing microspores (from 2 to 3 mm buds) was filtered through 45 μm nylon mesh. After two washes, as described by Albani *et al.* (1990), the pellet was frozen in liquid nitrogen and stored at -80 °C if not immediately used for RNA extraction. The purified male gametophytes from the different classes of bud were disrupted by 3 cycles of pressurization (at 110 bar) depressurization in a mini-bomb (Bioblock Scientific, Illkirch, France) in 50% RNA extraction buffer and 50% phenol before performing RNA extraction as previously reported (Dean *et al.*, 1985).

Poly(A)$^+$ RNAs were purified from total RNA using the mRNA purification kit from Pharmacia Biotech.

Total RNA was extracted from young rapeseed seedlings, total fertile and male-sterile buds, pistils, sepals and petals, roots, leaves and stems as described above.

cDNA library construction and cDNA subtraction

The procedure used for the isolation of organ-specific cDNA was based on an unpublished protocol from S. Lok and D.C. Baulcombe (Sainsbury Laboratory, John Innes Centre for Plant Science Research, Norwich, UK). It is the result of several reports on ligation-mediated PCR (Mueller and Wold, 1989), direct incorporation of biotin nucleotide during PCR (Lo *et al.*, 1988) and differential removal of biotinylated DNA by a streptavidin/phenol extraction procedure (Sive and St John, 1988; Wang and Brown, 1991) and was modified as follows.

The blunted 'tracer' cDNA (T) and the 'driver' cDNA (D) were synthesized from 3 μg of poly(A)$^+$ RNA from microspores and male-sterile buds (Ogu-INRA) (Gourret *et al.*, 1992), respectively, using a kit from Pharmacia Biotech. An aliquot of the tracer cDNA was ligated to the *EcoRI/NotI* adaptor according to the kit's instructions and inserted into the

*Eco*RI site of λgt10 and plated on non-permissive *Escherichia coli* strain C600 Hfl− using Gigapack III packaging extract (Stratagene). The titre of the library was estimated at 1.7×10^7 pfu/μg of λgt10 DNA.

A 200 ng portion of T and D cDNA (0.5 to 1 pmol of ends) was ligated to 280 pmol of the T (AATTCGCCATGGATCTAGACC / pGGTCTAGATCCATG) and D (ATCAGTGATCGGCATGAGCTCG / pCGAGCTCATGCCGA) specific linkers. A 50 ng portion of T and D DNA was then amplified for 12 cycles of PCR for the T (using the top T primer) and 30 cycles for the D using 50 nM Bio-11-dUTP (Sigma) and the top T primer. A 150 ng portion of amplified T was then subtracted with a 20-fold excess of biotinylated D for 24 h at 68 °C. The biotinylated DNA was then removed by the streptavidin/phenol extraction method. The subtraction step was repeated 2 times under the same conditions. 1/6 of the total subtracted tracer-driver (T-D) DNA was then amplified for 35 cycles using the T primer and the result was quantified on a gel. We chose to not directly clone the subtracted product as done by Rubinelli *et al.* (1998) but to use it as a probe to screen the microspore cDNA library in order to compensate the representational bias introduced by PCR.

Differential screening

A 20 ng portion of amplified subtraction product (T-D) and unsubtracted T cDNA were labelled at high specific activity with [^{32}P] dCTP with an oligolabelling kit (Pharmacia Biotech) (Feinberg and Vogelstein, 1985) and hybridized on duplicate filters (colony/plaque Screen filters, NEN Research Products, DuPont) containing approximately 20 000 plaques of the microspore cDNA library at 65 °C in 6× SSC for 24 h. The filters were washed in three steps of 30 min in 2×, 1× and 0.1× SSC with 0.1% SDS at 65 °C.

Only the clones showing a higher signal when hybridized with the T-D cDNA probe than with the T cDNA probe were selected and isolated. The accuracy of this choice was confirmed by a second round of hybridization realised in the same conditions on filters containing only the selected clones. Positive cDNA clones were purified and λDNA was extracted as previously described (Albani *et al.*, 1990). cDNA inserts were isolated after *Not*I or *Eco*RI digestion and subcloned in pBluescript SK− plasmid (Stratagene) digested by *Not*I or *Eco*RI.

Northern blot hybridization

A 10 μg portion of denatured total RNA was loaded in each lane of a 1.5% agarose gel containing formaldehyde and transferred onto Hybond-N membrane (Amersham) according to the manufacturer's protocol. Prehybridization and hybridization were carried out in a buffer containing 5× SSPE, 5× Denhardt's solution, 100 μg/ml sonicated salmon sperm DNA, 1% SDS and 50% deionized formamide at 42 °C for 6 h and 20 h, respectively. The selected cDNA fragments were gel-purified, labelled with [^{32}P] dCTP and used as probes for hybridization. The filters were washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (5×, 2× and 0.1× SSPE).

Genomic DNA library screening

350 000 recombinant clones (average size 15 kb) (three *B. napus* genome equivalents) of a genomic DNA library of *B. napus* cv. Bridger in bacteriophage λ EMBL-3 (Clontech, USA) were plated with *E. coli* LE392 as host. The entire M3 cDNA was used as a [^{32}P] dCTP-labelled probe for screening. Hybridization and washing were carried out with standard techniques (Sambrook *et al.*, 1989).

Determination of the 5′ end of mRNA

The method was based on PCR using the 5′-AmpliFINDER RACE kit from Clontech.

Two antisense primers were designed from the *BnM3.4* coding sequence: P1, 5′-TGATGACTCTAGT TCTGTTGCTGTG-3′ and P2, 5′-TGGAATTCGTTCT GTTGCTGTGACTTTGGATGT-3′, and we optimized the annealing temperature at 55°C for the step of PCR amplification. The experiment was performed with 2 μg of mRNA extracted from *B. napus* microspores, from *B. napus* floral buds and from *B. napus* leaves for the negative controls. Thereafter PCR products were digested by *Eco*RI and cloned into pBluescript SK− for sequencing.

Genomic DNA isolation and Southern blot hybridization

Genomic DNA was purified from seedling leaves using a standard procedure (Dellaporta *et al.*, 1983) followed by standard CsCl gradient centrifugation. Southern analysis was performed by digestion of 10 μg of genomic DNA, electrophoresis of DNA

fragments on a 0.8% agarose gel and blotting onto Hybond-N membrane using standard methods (Sambrook *et al.*, 1989). The M3 cDNA fragment was gel-purified and labelled with [^{32}P] dCTP as described previously. A Southern blot was hybridized overnight at 65 °C in 6× SSC and washed at 65 °C in three 15 min steps in 0.1% SDS and decreasing salt concentrations (6×, 2× and 0.1× SSC).

Plasmid construction

Recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989) to construct a transcriptional fusion bringing the expression of the *uidA* (commonly named *gus*) reporter gene under the control of the *BnM3.4* promoter in binary vector.

A 'promoterless' *gus* gene cassette, containing the *gus* coding sequence (Jefferson *et al.*, 1986) and the nopaline synthase polyadenylation site (*nos*-ter; Bevan *et al.*, 1983) was cloned into pBluescript SK– (Stratagene, USA), leading to pAF1opt. A 2056 bp *Bam*HI-*Nsp*V fragment (Figure 3) from the genomic clone *BnM3.4* was cloned into *Bam*HI-*Cla*I sites of the plasmid pAF1opt, generating pJD51. The chimaeric gene was then excised from pJD51 and introduced into the binary plasmid pEC2 (Cartea *et al.*, 1998) in inverse orientation with respect to the Basta resistance gene, generating pJD101. The promoterless *gus* gene cassette was also transferred into pEC2 to generate the negative control binary plasmid pAF100. The binary plasmids pAF100 and pJD101 were introduced into *Agrobacterium tumefaciens* strain C58C1 (Koncz and Schell, 1986) by electroporation (Nagel *et al.*, 1990; Singh *et al.*, 1993). The recombinant genes were introduced into *A. thaliana* by *in planta* infiltration (Bechtold *et al.*, 1993).

In situ *hybridization*

The *BnM3.4* coding sequence was cloned in both orientation in pGEM-3Zf(+) (pJD6 and 7). After *Sma*I linearization, these two plasmids were used to synthesize digoxigenin-11-rUTP-labelled probes using the Riboprobe Combination System T7 kit from Promega.

B. napus floral buds were fixed in 4% formaldehyde, embedded in wax, and 8 μm-sections were prepared for *in situ* hybridization according to Jackson (1991).

Full-length RNA probes were alkaline-hydrolysed to 150 nt fragments and hybridized to sections at a concentration of 0.5 ng/ml per kb of probe. Hybridization was carried out in 210 μl for a sandwich of two slides at 50 °C in 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 0.3 M NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA, 1 mg/ml yeast transfer RNA. Slides were washed in several baths of 0.2× SSC at 55 °C for 1 h, followed by two rinses of 5 min each with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (NTE buffer), and treated with 20 mg/ml RNase A in this buffer at 37 °C for 30 min. The slides were then washed again in NTE buffer and 0.2× SSC as described above, and finally washed in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7) for 5 min. Immunological detection of the hybridized probe was carried out as described in the Boehringer digoxigenin-nucleic acid detection kit with some modifications. Slides were incubated with gentle agitation for 1 h in 0.5% blocking agent (Boehringer) in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) followed by 1 h in 1% bovine serum albumin, 0.3% Triton X-100 in buffer 1 (buffer 2). This was followed by a 1 h incubation in dilute antibody conjugate (Boehringer) (1:1250) in buffer 2 and 4 washes of 15 min each in buffer 2. Slides were briefly washed in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ and incubated for 12–48 h in 0.34 mg/ml nitroblue tetrazolium salt and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. The colour reaction was stopped with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and sections were passed through an ethanol series before mounting in mounting medium from Sigma.

Histochemical GUS assays

Histochemical assays for GUS activity were conducted according to the protocol described previously by Jefferson *et al.* (1987) with some modifications. Fresh tissue samples were fixed in a solution containing chloroform, 95% ethanol and water in proportions 3:6:1 and 0.1% Triton X-100 under vacuum (−93.3 kPa) for 1 min. Then, tissue samples were washed twice in 50 mM potassium phosphate buffer, pH 7.0. GUS staining was performed by vacuum infiltration (3× 10 min at −93.3 kPa) in the GUS stain solution (Jefferson *et al.*, 1987) and by incubating at 37 °C overnight. The plant material was then cleared by rinsing with 70% ethanol and the samples were examined and photographed under a Diaplan type 307-148.002 microscope (Leitz, Wetzlar, Germany).

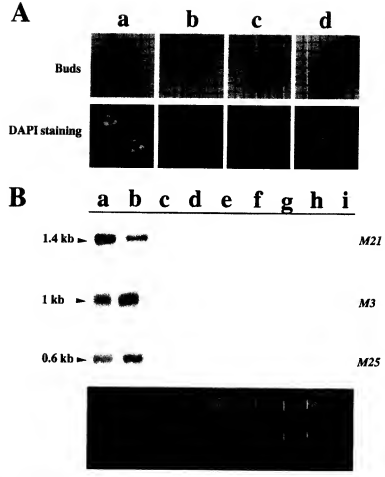

Figure 1. Correlation between the size of rapeseed flower buds and microgametophytic stage of development. Expression pattern of the M 21, M3 and M25 clones isolated by subtraction and differential screening of the microspore cDNA library. A. DAPI staining of the four different developmental stages during male gametogenesis (in the lower part) and the corresponding buds (in the upper part). a, DAPI-stained tetrads and earlier stages (not shown) contained in buds of length strictly below 2 mm; b, microspores contained in buds of 2–3 mm; c, bicellular pollen contained in buds of 3–4 mm; d, tricellular pollen contained in buds of 4.5 mm and more. Each square is 1 mm × 1 mm. B. Northern blot experiments hybridized with M21, M3 and M25 cDNA clones as probes. Total RNA (10 μg) from (a) meiocyte/tetrads, (b) microspores, (c) young (binucleate) and (d) mature (trinucleate) pollen, (e) sepals, (f) petals, (g) wild type and (h) CMS buds, and (i) *in vitro* grown seedlings were loaded on the gel. The picture of the corresponding ethidium bromide stained gel is presented underneath.

The pistil length was used as the unique criterion for identification of the stages of microsporocyte development in *A. thaliana*. Based on results of Bowman (1994), the correlation between the pistil length (pl) and the stages of microsporocyte development was further studied (D. Vezon, personal communication) and we found that a pl of 0.3-0.4 mm corresponds to meiosis; pl 0.5 mm corresponds to the end of meiosis and tetrad microspores; pl 0.75 corresponds to free microspores; pl 1.25 mm corresponds to the first pollen mitosis and bicellular pollen; pl 1.50 mm corresponds to the second pollen mitosis and tricellular pollen.

Results

Isolation of B. napus *cDNAs encoding sequences expressed early during pollen development*

Cytological studies (Scott *et al.*, 1991) have shown that the stages of male gametogenesis within the anther are highly correlated to bud length and that gametogenesis is synchronised in all anthers of one flower bud.

The cytological studies allowed us to establish that, in the growth conditions we used, rapeseed buds of length strictly below 2 mm contained mainly meiocytes and tetrads, 2 to 3 mm long buds contained

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M3      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
M3.21   TCTTGCTATG ATTTTCTTCA TAAGATGTGT CACATCCAAA GTCACAGCAA CAGAACTAGA GTCATCAACT AACCAAGAGC                          80

M3      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
M3.21   TCTTCCTATC GCGGCACTTG CCTCGCTTTC ACCCCAAGCC ACATTGGCCG TTCTGTGGCT CCGGAAAAGC CTTCCCTGCA                        160

M3      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
M3.21   GGCCACTTCC GACCAACTCC GTTCCATCTG CCACAGGAAG TCACCAGATG CTTGTCCGAC AAGAAGGAGG TAGGTACATG                        240

M3      .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... 
M3.21   TTTTGATGAT ATCGTTGAGA CTTTCTTCAC CAGGAAAGCC GTTATTGGAT CGAATGTTG CGCCGCGATC AAGAAGATGA                        320

M3      .......... .......... ...TTGGAT CTTTCCATGA CCCCTTCTTG ACCGGCTATG TCAAGCTACA TTGCTCCACC
M3.21   ACAAAGATTG TGAGAAGACC GTCTTTGGAT CTTTCCATGA CCCCTTCTTG ACAGCTATG TCAAACTACA TTGCTCCACC                        400

M3      GTTGTTGGAT CTACTTCACC TCCTCCTTCA CAGGCTCCTT TACATGCTCC TTCTTCACAG GCTCCTTCAC ATGCTCCTTC
M3.21   GTTGTTGGAT CTACTTCACC TCCTCCTTCA ---------- ---------- ---------- ---------- ----------                        480

M3      ACATGCTCCT TCACAGGCTC CTTTAAATGC TCTTTAAAT GCTCCTTTAC ATGCTCCTTT ACATGCTCCT TCACAGGCCC
M321    ---------- ---------- ---------- ---------- ---------C ATGCTCCTTC ACAGGCTCCT TTACATGCTC                        560

M3      CTTCACAGGC CCCTTCACAG GCCCCTTTAC ATGCTCCTTT ACTGCCCCCT TCGCAGGCTC CTTCACCGGC TCAGTGA-TT
M3.21   CTTCACAGGC TCCTTTACAT GCCCCTTCAC AGGCTCCTTT ACTGCCCCCT TCACAGCCTC TCCCACCGGC TCAGTGATTT                        640

M3.     TAGCTATTTG ATAGAATTAC TCAAGTAATG ATGCCCTAGG GAGTTTGAGT TTTTCTCGTG TTTTAAAGTT TTGTGTTTAT
M321.   TAGCTATTTG TTAGAATTAT TCAAGTGTTG ATGTCCTAGG GAGTTTTAGG TTTT-TCTTG TTTTAAAATT TTGTGTTTAT                        720

M3      TTTGAGAAAA CCGTCTTTGG ATTTTAACTT CACTTTGATT TTTTCCCTTA TACAATTTAA ATTTAGAGTT TACTTATTAA
M3.21   TTTGAGAAAA CCGTCTTTGG ATCTTAACTT CACTTTGATT TTTT-CCTTA TACAA..... .......... ..........                        800

M3      TTTTATAAAT TAGATGGTAC TAAGTTTTTA TCATAATAAA A
M3.21   .......... .......... .......... .......... .                                                                  841
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Figure 2. Comparison of M3 and M3.21 cDNA sequences. Numbers at the right margin refer to the nucleotide position. Dashed lines indicate gaps and underlined sequences represent imperfect repeated motifs found in the M3 and M3.21 sequences. The stop codons are shown in bold type.

mainly microspores, 3 to 4 mm long buds contained mainly bicellular pollen grains and buds of 4.5 mm and more in length contained mature tricellular pollen grains (Figure 1A). RNAs were prepared from male gametophytes purified from buds at these four stages. To achieve this we had to develop a new technique (see Materials and methods) to lyse the microspores which have extremely resistant cell walls. To obtain a driver for the subtraction procedure, we isolated RNA from whole male-sterile flower buds (Ogu-INRA). These buds are identical to those of wild type except that male gametophyte development is arrested before the tetrad stage and consequently they do not contain any microspores (Gourret *et al.*, 1992).

Poly(A)$^+$ RNAs from microspores were converted to cDNA, of which a fraction was used to construct a cDNA library. To generate probes enriched for microspore-specific transcripts, another fraction of the above microspore cDNA was then subtracted several times by hybridization with cDNA made from male-sterile buds (see Materials and methods). We then screened the microspore cDNA library using subtracted and primary probes. After two rounds of such successive screens, we retained 32 potential candidates that showed increased hybridization to the substracted probe with respect to the unsubtracted microspore cDNA probe.

The expression pattern of selected clones was compared in more detail by slot blot (data not shown) and by northern blot analyses with total RNA isolated from the four same developmental stages of male gametophytyte as above, entire wild-type and male-sterile buds, sepals, petals and *in vitro* grown seedlings or leaves. These expression studies allowed us to classify these genes into four groups.

The first group consists of 13 clones whose expression is strictly confined to the male gametophyte and high in the microspore. The second group contains 13

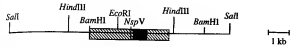

Figure 3. Analysis of the *BnM3.4* gene. Partial restriction map of the *BnM3.4* genomic clone. The striped box represents the sequenced region of the *BnM3.4* genomic clone; the black one the coding sequence of the *BnM3.4* gene. The two *Sal*I sites border the 10 kb insert.

clones whose expression is high in the microspores but not restricted to the male gametophyte since they are also expressed in other sporophytic tissues. The third and fourth groups correspond respectively to 2 clones having a weak expression restricted to microspores and 4 clones showing weak expression in all tissues tested.

We characterized further three cDNAs belonging to the first group of genes expressed early and specifically during male gametophyte development. Northern blot analyses of these clones are shown in Figure 1B. The M21 gene detected a mRNA of 1.4 kb (encodes potentially a novel protein), which is expressed at the meiocyte and tetrad stages but its mRNA level remains high in the microspore. The M25 cDNA detected a 0.6 kb mRNA (encoding a putative SKP1-like protein (Connelly and Heiter, 1996; Krek, 1998)), mainly in the microspore but expression was also detected at meiocyte/tetrad stages and in bicellular pollen. The M3 cDNA detected a 1.0 kb mRNA in microspores but expression was also evident in meiocyte/tetrad stages. We have chosen to focus on this particular clone for further studies because extensive hybridization studies using a variety of sporophytic tissues (data not shown) suggested that the M3 mRNA is highly early stage microspore-specific.

Analysis of the nucleotide sequences of M3 and M3.21 cDNAs

The M3 cDNA has a length of 497 bp. It was smaller than the 1.0 kb mRNA detected on northern blots, so M3 is likely to be a partial cDNA. A second screening of the microspore cDNA library was performed using M3 as probe. A longer but still partial clone, named M3.21, of 674 bp was then isolated. The expression pattern of the M3.21 cDNA was checked by northern blot (data not shown) and appeared to be identical to the M3 pattern. Both cDNA clones, M3 and M3.21, were subcloned into pBluescript SK− and sequenced. Alignment of their nucleotide sequences shows that the M3 cDNA differs essentially from M3.21 by an insertion of 99 bp constituted by three repeats of the same motif (Figure 2).

M3 cDNA and the corresponding BnM3.4 *genomic clone: DNA sequence comparisons and analyses*

The M3 cDNA was used as a probe to screen a *B. napus* genomic DNA library in order to isolate the corresponding gene. One of the isolated clones, BnM3.4, with an insert size of 10 kb, was selected for further characterization (Figure 3). A 2.9 kb sequence surrounding the *BnM3.4* gene was deposited in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AF136223. Two putative in-frame translation start codons, separated by 114 bp and leading to 2 ORFs of 712 bp and 598 bp, were detected (respectively at 1971 and 2085 nucleotides from the start of the sequence). Comparison of the M3 cDNA sequence with the sequence of the shortest ORF of the genomic clone revealed 1 difference out of 293 bp of aligned coding sequence and 7 differences out of 203 bp of aligned 3′-downstream region. These sequence differences between genomic and cDNA clones were repeatedly found in independent sequencing experiments and might be due to allelic variations between the different *B. napus* varieties used in the construction of the cDNA and genomic libraries. No intron was detected in the region covered by the cDNA and no intron/exon junctions were found in the rest of the ORF using a splice site prediction program (Hebsgaard *et al.*, 1996). A putative polyadenylation signal (Joshi, 1987b) was found 197 bp downstream of the stop codon. Because we are ultimately interested in the regulation of pollen and anther-specific genes, we first determined by 5′ RACE which of the two potential start codons in M3/M3.21 are used exclusively or preferentially. Sequences of 12 PCR fragments revealed two groups of products. The first group contained seven sequences identical to the *BnM3.4* gene (PCR1 in Figure 4). The longest PCR product showed that the transcription start of the *BnM3.4* gene is a G (underlined on Figure 4), localized between the two potential translation starts. Primer extension experiments (data not shown) indicated approximately the same nucleotide for the transcriptional start site. The length between the transcriptional start site and the second ATG (64 bp), is in agreement with the predominant length of leader sequences (40–80 nucleotides) in plant genes (Joshi, 1987a). Moreover, the context of the second ATG agrees well with the consensus sequence (AAorCAAUGGC) proposed for

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BnM3.4    -21 AACCCTAACG CCTTACCACT CGATAACCAT CAAAACTTTT CTTCTCGTTT 29
PCR1          ..........  ..........  .GATAACCAT CAAAACTTTT CTTCTCGTTT
PCR2          ..........  .....ACT CAATAACCAT CAAAACTTTT CTTCTCGTTT

BnM3.4      CGCTAACTCA AGGCTTCGAA AAGTAAAAAA AACAATGAAG AATGTCACAC 79
PCR1        CGCTAACTCA AGGCTTCGAA AAGTAAAAAA AACAATGAAG AATATCACAC
PCR2        CGCAAACTCA AAGCTTCAAA AAGT---AAG AACAATGAAG AATGTCTCAC

BnM3.4      TTGTTCTTGC TATGATCCTC TTCTTAAGCT GTGTCACATC CAAAGTTACA 129
PCR1        TTGTTCTTGC TATGATCCTC TTCTTAAGCT GTGTCACATC CAAAGTTACA
PCR2        TTGTTCTTGC TATGATCCTC TTCTTAAGCT GTGTCACATC CAAAGTCACA

BnM3.4      GCAACAGAAC TAGAGTCATC AACTAACCAA GAGCTCTTCC 169
PCR1        GCAACAGAAC  .......... .......... ..........
PCR2        GCAACAGAAC  .......... .......... ..........
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Figure 4. Alignment of the *BnM3.4* gene sequence and the longest representatives of the two types of products PCR1 and PCR2 from the 5′ RACE PCR on mRNA from *B. napus* microspores. Dashed lines indicating gaps and nucleotide differences are reported in bold type in PCR2. The transcription start site (G) is underlined (in *BnM3.4* and PCR1) and the ATG codon is in bold type.

dicotyledon nuclear genes by Joshi *et al.* (1997). A clear TATA box sequence (TATATATA) was identified 31 nucleotides upstream of the start of transcription (Messing *et al.*, 1983). The second group of 5′ RACE PCR products (PCR2 in Figure 4) presents several nucleotide differences with respect to the first group. The detection of this second group proves that a second gene closely related to *BnM3.4* exists and is expressed in the microspore at approximately the same level as *BnM3.4*. It should not correspond to allele variation of *BnM3.4* because cv. Brutor used to make the cDNA library and the 5′ RACE experiments is an inbred line. This gene may correspond to the M3.21 cDNA but M3.21 is too short at its 5′ end to enable us to compare it to the PCR2 sequences and determine whether they come from the M3.21 type mRNAs. To ascertain this, a Southern blot analysis was performed on total DNA extracted from *B. napus* cv. Brutor and digested by *Bam*HI, *Eco*RI and *Hind*III. The DNA gel blot was hybridized with ^{32}P-labelled M3 cDNA at high stringency. As shown in Figure 5, in addition to the 5 kb *Hind*III and 5.5 kb *Bam*HI fragments corresponding to the *BnM3.4* genomic clone, the *B. napus* genome contained other fragments which were clearly recognized by the M3 cDNA probe, consistent with the idea of at least one other expressed homologue in *B. napus*. These homologous genes will be called the *BnM3* family.

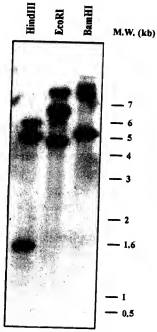

Figure 5. Southern blot analysis of genomic DNA from *B. napus* (cv. Brutor). A 10 μg portion of genomic DNA was digested with *Bam*HI, *Eco*RI and *Hind*III. Hybridization was performed with ^{32}P-labelled M3 cDNA as probe. DNA size standards (in kb) are shown to the right of the figure.

Characteristics of the putative BnM3.4 protein

The *BnM3.4* gene encodes a putative 218 amino acid protein with a molecular mass of 23.4 kDa and a predicted pI of 8.8. The predicted BnM3.4 protein shows limited similarity with two potential *A. thaliana* products of genomic clones MKP11 (accession number AB005238) and K19P17 (AB007644). The N-terminal 152 aa of BnM3.4 is 55% identical (69% similarity) to MKP11, while the carboxyl portion of BnM3.4 is 45% identical (63% similarity) to K19P17. These two translated sequences do not correspond to two exons of the same gene since they are separated

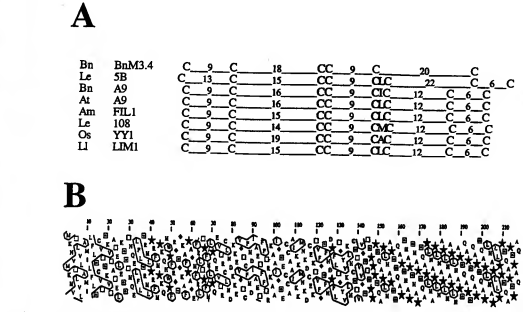

Figure 6. BnM3.4 protein characteristics. A. Cysteine pattern alignment of the BnM3.4 protein with sequences of seven stamen-specific genes. The number of amino acids between cysteine residues is given, but the spacing is not proportional. Le 5B, *Lycopersicon esculentum* TomA5B (Aguirre and Smith, 1993); Bn A9, *Brassica napus* A9; At A9, *Arabidopsis thaliana* A9 (Paul *et al.*, 1992); Am FIL1, *Antirrhinum majus* FIL1 (Nacken *et al.*, 1991); Le 108, *Lycopersicon esculentum* 108 (Chen and Smith, 1993); Os YY1, *Oryza sativa* YY1 (Hihara *et al.*, 1996), Li LIM1, *Lilium longiflorum* LIM1 (Kobayashi *et al.*, 1994). The amino acid sequences were deduced from DNA sequences. B. HCA (Hydrophobic Cluster Analysis) representation of the BnM3.4 protein. Amino acids are represented with one-letter symbols except for: proline (stars); glycine (diamonds); threonine (open squares); and serine (squares with dot). Hydrophobic clusters are endorsed by a black line.

by 16 Mb on the chromosome. The functions of these two putative *A. thaliana* gene products are not known.

The putative BnM3.4 protein, however, has three recognizable structural domains. The residues from 1 to 23 are hydrophobic, characteristic of a signal peptide, and a cleavage site between the alanine at position 23 and the threonine at position 24 can be predicted according to the rules of von Heijne (Nielsen *et al.*, 1997). From these features, the protein may be secreted and this N-segment may help the protein to cross or to interact with the membrane. The central part of the protein (residues 25–143) is hydrophilic. The spacing of six particular cysteines in this segment resembles that found in various stamen-specific proteins: TomA5B (Aguirre and Smith, 1993), FIL1 (Nacken *et al.*, 1991), A9 (Paul *et al.*, 1992), 108 (Chen and Smith, 1993), YY1 (Hihara *et al.*, 1996) and LIM1 (Kobayashi *et al.*, 1994). The possible relationship among these cysteine-rich proteins and the BnM3.4 protein is best illustrated by showing only their pattern of cysteine residues (Figure 6A). Although the BnM3.4 protein presents a shorter pattern than the others, the position and spacing of the cysteine residues are very similar.

The C-terminal domain consists of 14 repeats related to the motif P-[SL]-[HNQ]-A. Analysis of this domain by the Hydrophobic Cluster Analysis program (available at: www.lmcp.jussieu.fr/~soyer/www_hca/hca-form.html) suggests that the proline residues in the motifs could form the backbone of a rigid helical structure (Figure 6B).

In situ *analysis of the expression of the* BnM3 *genes family*

The pattern of *BnM3* expression during the four stages of gametogenesis (see above) was examined in bud tissues of *B. napus* by *in situ* hybridization using the *BnM3.4* antisense transcript as a probe. As at least two homologous genes are present in the *B. napus* genome, the hybridization pattern may reflect the expression pattern of (at least) these two genes. The *BnM3* transcripts were not detectable in anthers with pollen mother cells undergoing meiosis (data not shown), nor in tetrads (Figure 7A), nor in bicellular pollen (Figure 7G), nor in tricellular pollen (Figure 7I). However, at the developmental stage where rapeseed buds are 2.5 mm, a strong hybridization signal was detected in the microspore (Figure 7C). At high magnification

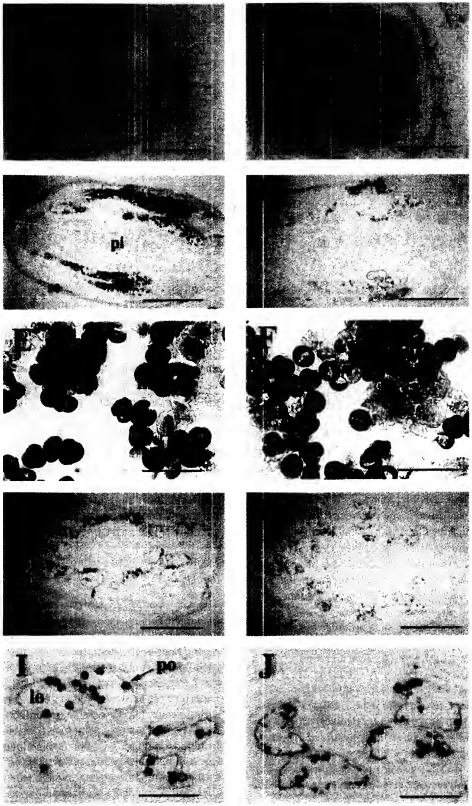
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Figure 7. BnM3.4 expression in floral buds of *B. napus*. *In situ* hybridization of DIG-labelled antisense (A, C, E, G and I) or sense (B, D, F, H and J) *BnM3.4* RNA probe to *B. napus* floral bud sections. A and B. Transverse sections of 1.5 mm long buds containing tetrads (bar = 4 μm). C and D. Longitudinal sections of 2.5 mm long buds containing microspores (bar = 10 μm). E and F. Magnification of anthers from transverse sections of 2.5 mm long buds (bar = 0.8 μm). G and H. Transverse sections of 3.5 mm long buds containing bicellular pollen (bar = 10 μm). I and J. Transverse sections of anthers from 4.5 mm long buds containing tricellular pollen (bar = 4 μm). Abbreviations: an, anther; lo, locule; mi, microspore; pi, pistil; po, mature pollen; se, sepal; ta, tapetal cell.

(Figure 7E), the *BnM3* transcripts are clearly visible by a deep staining inside the microspores. No hybridization signal was detected in any male gametophyte tissue at any stage of development with the sense probe (Figure 7B, D, F, H, J). The lack of hybridization of some microspores with the antisense probe is probably an artefact caused by a partial section of the pollen grain or a loss of cytoplasm during the pretreatment of slides. Both the sense and the antisense probes allowed us to detect a weak hybridization signal in tapetal cells that could be considered as a background signal not specific to the *BnM3.4* transcripts.

The BnM3.4 *promoter: regions of homology and functional analysis*

A detailed analysis of the *BnM3.4* promoter sequence revealed the presence of several regions having possible regulatory functions. Two 14 bp repeats (ttaaaagtggaac) are located from −1852 to −1780 from the transcription start. A 14 bp palindromic sequence (ttagaatattctaa) is located at −307 while another 12 bp palindrome (ttttaattaaaa) starts at −213. These motifs have not been detected in any other pollen-specific gene. However, the motif 'tcatttt', located between −593 and −517 and the reverse motif 'aaatga', located at −1566 in *BnM3.4* upstream regions were already described for other pollen-specific genes like *NTP303* (Weterings *et al.*, 1995), *NTM19* (Oldenhof *et al.*, 1996) and *Bp10* (Albani *et al.*, 1992). A 2 kb region of this 5′-flanking sequence, containing these different regions was transcriptionally fused with the reporter *gus* coding sequence. The resulting construction (pJD101, see Materials and methods) was introduced into *A. thaliana*. This species was chosen for these experiments because it belongs to the same botanical family as *B. napus*, but it can be more easily transformed and has a shorter life cycle. GUS staining was first performed on the inflorescences of fifteen independent transgenic *A. thaliana* T_1 lines (JD101 series) transformed with this gene. They all showed qualitatively the same expression patterns in the course of gametophyte development (not shown). The progeny (T_2) of T_1 plants with insertion of the transgene(s) presumably at a single locus was chosen for further analysis. The results of histochemical analysis of *gus* expression for individual hemizygous and homozygous progeny of one of them, JD101.42, are shown in Figure 8. This transformant presents one insertion according to the genetic test (275 phosphinotricine resistant plants out of 380 seedlings; $\chi^2 = 1.40$; $P < 0.05$).

In all the transgenic JD101 plants and their progeny studied, no GUS activity was detected in flower organs other than the stamen as shown in Figure 8A for one hemizygote progeny of the JD101.42 plant. GUS activity was not detected in any organ of seedling (Figure 8I) or older plants (not shown), or in siliques (Figure 8J). In comparison, plants transformed with the control construction pAF100 (*gus* without promoter) showed no GUS activity (Figure 8A, inset). The presence of 50% of blue pollen and 50% of unstained pollen in anthers of the hemizygous JD101.42 plant (Figure 8H) confirms that the *BnM3.4* promoter is only activated in gametophytic cells i.e only the transgenic pollen shows GUS activity.

The temporal pattern of *gus* expression was determined with homozygous JD101 progeny with the aim of precisely determining the developmental stage where the reporter gene is activated. At the tetrad stage of male gametophyte, no GUS activity was detected (Figure 8B); it only appears at a high level at the beginning of the microspore stage (Figure 8C). As shown in Figure 8E, high levels of GUS activity were detected in pollen. There seems to be a low level of the GUS product in the locule which may be the result of diffusion, but no activity was present in the sporophytic tissues of the anther such as the epidermis, the endothecium and the tapetum. The GUS expression level stays constant until the mature tricellular pollen stage (Figure 8D, 8F and 8G) but it is difficult to know if it is due to the longevity of the GUS protein or transcription activity in *Arabidopsis*.

At least for the first stages of male gametophyte development, the reporter gene has the same expression pattern as intact genes.

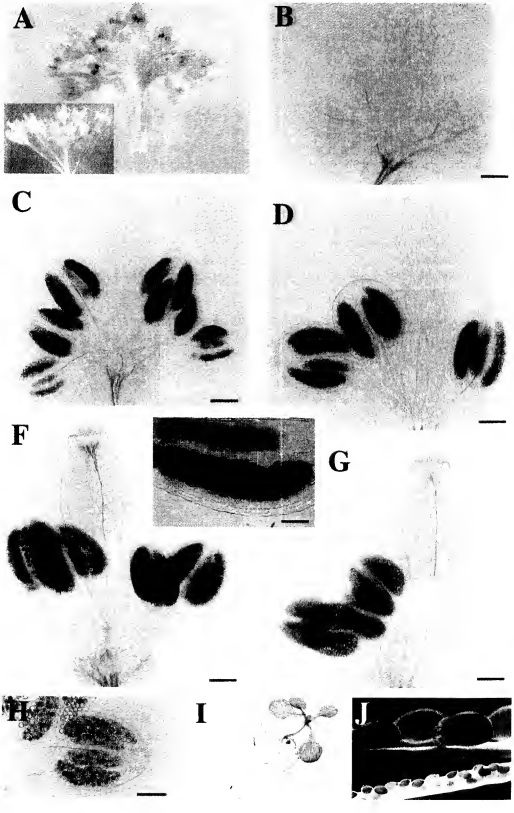
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Figure 8. Histochemical analysis of *gus* expression in transgenic *A. thaliana* JD101 plants. The following plant materials from homozygous and hemizygous JD101.42 transgenic plants and from a AF100 transgenic plant were examined for GUS activity as described in Materials and methods. A. Inflorescence from a 5-week old homozygous pJD101 plant and (in the inset) the inflorescence from a 5-week old AF100 plant (negative control plant). B–G. Developmental series of buds from the stained inflorescence presented above. Sepals and petals were removed from the bud in order to measure the pistil length more easily and to improve the visualization of GUS in anthers. The stages of male gametophyte development were determined according to the pistil length as described in Materials and methods. B, tetrad stage; C, early microspore stage; D, end of microspore stage; E, locule of one of the anthers of the bud shown in D, magnified 6× relative to Figure D (bar represents 27 μm); F, bicellular pollen. g, tricellular pollen in anthers at anthesis. In B, C, D, F and G, bars represent 90 μm. H. Anther of a hemizygous plant, at the microspore stage (bar represents 70 μm). I. Ten-day old seedling. J. Open siliques and seeds at two different magnifications.

Discussion

This paper describes a method in order to isolate cDNAs from rapeseed that are preferentially expressed during early male gametophyte development. Expression of several of these genes appears to be highly specific to microspores and we have analysed one of these, called *BnM3.4*, in detail. This gene corresponds to the M3 cDNA isolated, but another cDNA was isolated from the microspore cDNA library using M3 as a probe. This longer cDNA (M3.21) is homologous to M3 except that it shows a deletion of 99 bp in the putative coding region. As these two cDNA cross-hybridize in the hybridization conditions used, we can conclude that at least two genes (the *BnM3* family=*BnM3.4* + related gene(s)) are present in the *B. napus* genome. This is also supported by the results of 5′ RACE and Southern blot experiments.

The expression of these genes was studied both by northern blot and *in situ* hybridization. By northern blot analysis, the transcripts were observed mainly in microspores and also in total RNA extracted from buds of length strictly below 2 mm, that contain a majority of tetrads and meiocytes. The developmental synchrony between all the male gametophytes of an anther is not perfect and some free microspores already appear among tetrads in buds below 2 mm, so the hybridization of M3 probe to RNAs from pollen extracted from buds below 2 mm may be due to the early presence of microspores in these buds. This hypothesis was confirmed by *in situ* hybridization experiments. In individual 1.5 mm long buds, where no microspores were found, no signal was detected. On the other hand, in 2.5 mm long buds, where microspores are the only developmental stage present, a strong signal was detected. In later gametogenesis stages, no further signal was detected. According to these results, we can conclude that the homologous genes of *BnM3* family are expressed specifically in the microspore of *B. napus*.

Like the other early-expressed gene isolated from tobacco, *NTM19* (Oldenhof *et al.*, 1996), *BnM3.4* does not show any homology to known genes. Although the deduced BnM3.4 protein does not show homology with the deduced NTM19 protein, they are both proline-rich proteins and contain a putative signal peptide. We can only speculate on the function and cellular localisation of the BnM3.4 protein with the help of prediction programs. Several stamen proteins of unknown function contain similar proline-rich domains (Chen *et al.*, 1993) that, despite differences in amino-acid composition, are structured in a very similar way. The role of the proline-rich motifs is not known, but one may speculate that they serve as some kind of anchoring structures in interaction with either components of the cytoskeleton or with structural components of the cell wall (Woessner *et al.*, 1994; Kröger *et al.*, 1996).

The comparison analysis of the *NTM19* and the *BnM3.4* promoter regions did not show any clear common domains which could indicate a role in the specificity of expression of these genes. Only the box 'tcattt' and its reverse sequence, 'aaatga', which has already been shown to be important for pollen-specific expression are present in both promoters. Even if the number and the precise location of these motifs are not conserved between the different promoter regions described (tobacco for *NTM19* and *NTP303*, rapeseed for *Bp10*), functional analysis using deletion and targeted mutagenesis experiments in microprojectile-mediated transient expression assays (Weterings *et al.*, 1995) showed clearly that one of these elements of the *NTP303* promoter constitutes a positive *cis*-regulatory element and functions by specifically enhancing transcription in pollen. This is the reason why we kept this promoter region of 2 kb for the heterologous expression experiments. Both analysis of more microspore-specific genes and promoter deletions using a reporter gene will be necessary to evaluate the influence of the different motifs in the spacial and temporal regulation of transcription of these genes.

The histochemical analysis of *gus* expression driven by the *BnM3.4* promoter (pJD101 construction) in transgenic *A. thaliana* plants showed a high level of GUS activity in male gametophytes but not in other floral and seedling tissues. Furthermore, GUS activity appeared at the microspore stage and stayed at a constant level during pollen development until pollen maturity. GUS being a very stable protein, the detection of GUS activity allowed us to define precisely the start point of *BnM3.4* promoter activity in *A. thaliana* but not its stop point. The spatial and temporal pattern of *gus* expression driven by the *BnM3.4* promoter in *A. thaliana* is in accordance with the established expression pattern of the gene in *B. napus*. These results suggest the presence of *trans*-acting factors in *A. thaliana* able to recognise and interact with the *cis*-regulatory elements of *BnM3.4* in the same manner as in *B. napus*.

Several of the genes isolated by this procedure belong to gene families (*BnM3.4, SKP1*-like genes) as seen with other transcriptional approaches (Twell, 1994). This strategy is complementary to the mutagenesis being undertaken in *A. thaliana* (Feldmann *et al.*, 1997; Bonhomme *et al.*, 1999). We now plan to compare the 5′-flanking regions of the other microspore-specific genes we have isolated and to try *BnM3.4* promoter deletion analysis in order to find *cis*-regulatory elements required for microspore-specific expression.

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References

Aguirre, P.J. and Smith, A.G. 1993. Molecular characterization of a gene encoding a cysteine-rich protein preferentially expressed in anthers of *Lycopersicon esculentum*. Plant Mol. Biol. 23: 477–487.

Albani, D., Robert, L.S., Donaldson, P.A., Altosaar, I., Arnison, P.G. and Fabijanski, S.F. 1990. Characterization of a pollen-specific gene family from *Brassica napus* which is activated during early microspore development. Plant Mol. Biol. 15: 605–622.

Albani, D., Sardana, R., Robert, L.S., Altosaar, I., Arnison, P.G. and Fabijanski, S.F. 1992. A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. Plant J. 2: 331–342.

Bechtold, N., Ellis, J. and Pelletier, G. 1993. *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.r. Acad. Sci. (Sér. III: Sci. Vie) 316: 1194–1199.

Bedinger, P.A., Hardeman, K.J. and Loukides, C.A. 1994. Travelling in style: the cell biology of pollen. Trends Cell Biol. 4: 132–138.

Bevan, M., Barnes, W.M. and Chilton, M.-D. 1983. Structure and transcription of the nopaline synthase gene region of T-DNA. Nucl. Acids Res. 11: 369–385.

Bonhomme, S., Horlow, C., Vezon, D., de Laissardière, S., Guyon, A., Férault, M., Marchand, M., Bechtold, N. and Pelletier, G. 1999. T-DNA mediated disruption of essential gametophytic genes in *Arabidopsis* is unexpectedly rare and cannot be inferred from segregation distortion alone. Mol. Gen. Genet, in press.

Bowman, J. (Ed.) 1994. *Arabidopsis*. An Atlas of Morphology and Development, Springer-Verlag New York, pp. 133–332.

Brander, K.A. and Kuhlemeier, C. 1995. A pollen-specific DEAD-box protein related to translation initiation factor eIF-4A from tobacco. Plant Mol. Biol. 27: 637–649.

Brown, S.M. and Crouch, M.L. 1990. Characterization of a gene family abundantly expressed in *Oenothera organensis* pollen that shows sequence similarity to polygalacturonase. Plant Cell 2: 263–274.

Bucciaglia, P.A. and Smith, A.G. 1994. Cloning and characterization of *Tag1*, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Mol. Biol. 24: 903–914.

Cartea, M.E., Migdal, M., Galle, A.M., Pelletier, G. and Guerche, P. 1998 Comparison of sense and antisense methodologies for modifying the fatty acid composition of *Arabidopsis thaliana* oilseed. Plant Sci 136: 181–194.

Chen, C.G., Mau, S.L. and Clarke, A.E. 1993. Nucleotide sequence and style-specific expression of a novel prolin-rich protein gene from *Nicotiana alata*. Plant Mol. Biol. 21: 391–395.

Connelly, C. and Heiter, P. 1996. Budding yeast SKP1 encodes an evolutionarily conserved kinetochore protein required for cell cycle progression. Cell 86: 275–285.

Chen, R. and Smith, A.G. 1993. Nucleotide sequence of a stamen- and tapetum-specific gene from *Lycopersicon esculentum*. Plant Physiol. 101: 1413.

Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedsbrook, J. 1985. Differential expression of eight genes of petunia ribulose bisphosphate carboxylase small subunit multi-gene family. EMBO J. 4: 3055–3061.

Dellaporta, J.L., Wood, J. and Hicks, J.B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1: 19–21.

Estelle, M.A. and Sommerville, C.R. 1987. Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol. Gen. Genet. 206: 200–206.

Feinberg, A.P. and Vogelstein, B. 1985. A technique for radiolabelling DNA fragments to high specificity. Anal. Biochem. 137: 266–267.

Feldmann, K., Coury, D. and Christianson, M. 1997. Exceptional segregation of a selectable marker (KanR) in *Arabidopsis* identifies genes important for gametophytic growth and development. Genetics 147: 1411–1422.

Gourret, J.P., Delourme, R. and Renard, M. 1992. Expression of *ogu* cytoplasmic male sterility in cybrids of *Brassica napus*. Theor. Appl. Genet. 83: 549–556.

Hanson, D.D., Hamilton, D.A., Travis, J.L., Bashe, D.M. and Mascarenhas, J.P. 1989. Characterization of a pollen-specific cDNA clone from *Zea mays* and its expression. Plant Cell 1: 173–179.

Hebsgaard, S.M., Korning, P.G., Tolstrup, N., Engelbrecht, J., Rouze, P. and Brunak, S. 1996. Splice site prediction in *Arabidopsis thaliana* pre-mRNA by combining local and global sequence information. Nucl. Acids Res. 24: 3439–3452.

Hihara, Y., Hara, C. and Uchimiya, H. 1996. Isolation and characterization of two cDNA clones for mRNAs that are abundantly expressed in immature anthers of rice (*Oryza sativa* L.). Plant Mol. Biol. 30: 1181–1193.

Jackson, D.P. 1991. *In situ* hybridization in plants. In: D.J. Bowles, S.J. Gurr and M. McPherson (Eds.), Molecular Plant Pathology: A Practical Approach, Oxford University Press, Oxford, pp. 163–174.

Jefferson, R.A., Burgess, S.M. and Hirsh, D. 1986. ß-glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA 83: 8447–8451.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Joshi, C.P. 1987a. An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucl. Acids Res. 15: 6643–6653.

Joshi, C.P. 1987b. Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. Nucl. Acids Res. 15: 9627–9640.

Joshi, C.P., Zhou, H., Huang, X. and Chiang, V.L. 1987. Context sequences of translation initiation codon in plants. Plant Mol. Biol. 35: 993–1001.

Kobayashi, T., Kobayashi, E., Sato, S., Hotta, Y., Miyajima, N., Tanaka, A. and Tabata, S. 1994. Characterization of cDNAs induced in meiotic prophase in lily microsporocytes. DNA Res. 1: 15–26.

Koltunow, A.M., Truettner, J., Cox, K.H., Wallroth, M. and Goldberg, R.B. 1990. Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224.

Koncz, C. and Schell, J. 1986. The promoter of TL-DNA gene $5'$ controls the tissue specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. 204: 383–396.

Krek, W. 1998. Proteolysis and the G1-S transition: the SCF connection. Curr. Opin. Genet. Devel. 8: 36–42.

Kröger, N., Bergsdorf, C. and Sumper, M. 1996. Frustulins: domain conservation in a protein family associated with diatom cell walls. Eur. J. Biochem. 239: 259–264.

Lo, Y.-M.D., Mehal, W.Z. and Kleming, K.A. 1988. Rapid production of free biotinylated probes using the polymerase chain reaction. Nucl. Acids Res. 16: 8719–8722.

Messing, J., Geraghty, D., Heidecker, G., Hu, N.T., Kridl, J. and Rubenstein, I. 1983. Plant gene structure. In: T. Kosuge, C.P. Meredith and A. Hollaender (Eds.), Genetic Engineering of Plants, Plenum, New York, pp. 211–227.

Mueller, P.R. and Wold, B. 1989. In vivo footprinting of a muscle-specific enhancer by ligation mediated PCR. Science 246: 780–786.

Nacken, W.K.F., Huijser, P., Beltran, J.-P., Saedler, H. and Sommer, H. 1991. Molecular characterization of two stamen-specific genes, *tap1* and *fil1*, that are expressed in the wild type, but not in the *deficiens* mutant of *Antirrhinum majus*. Mol. Gen. Genet. 229: 129–136.

Nagel, R., Masel, E.A., Birch, R.G. and Manners, J.M. 1990. Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. FEMS Microbiol. Lett. 67: 325–328.

Nielsen, H., Engelbrecht, J., Brunak, S., Von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Prot. Engng. 10: 1–6.

Oldenhof, M.T., de Groot, P.F.M., Visser, J.H., Schrauwen, J.A.M. and Wullems, G.J. 1996. Isolation and characterization of a microspore-specific gene from tobacco. Plant Mol. Biol. 31: 213–225.

Paul, W., Hodge, R., Smartt, S., Draper, J. and Scott, R. 1992. The isolation and characterisation of the tapetum-specific *Arabidopsis thaliana* A9 gene. Plant Mol. Biol. 19: 611–622.

Robert, L.S., Allard, S., Gerster, J.L., Cass, L. and Simmonds, J. 1993. Isolation and characterization of a polygalacturonase gene highly expressed in *Brassica napus* pollen. Plant Mol. Biol. 23: 1273–1278.

Ross, J.H.E. and Murphy, D.J. 1996. Characterization of anther-expressed genes encoding a major class of extracellular oleosin-like proteins in the pollen coat of Brassicaceae. Plant J. 9: 625–637.

Rubinelli, P., Hu, Y. and Ma, H. 1998. Identification, sequence analysis and expression studies of novel anther-specific genes of *Arabidopsis thaliana*. Plant Mol. Biol. 37: 607–619.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scott, R., Dagless, E., Hodge, R., Paul, W., Soufleri, I. and Draper, J. 1991. Patterns of gene expression in developing anthers of *Brassica napus*. Plant Mol. Biol. 17: 195–207.

Shen, J.B. and Hsu, F.C. 1992. *Brassica* anther-specific genes: characterization and *in situ* localization of expression. Mol. Gen. Genet. 234: 379–389.

Singh, A., Kao, T. and Lin, J.J. 1993. Transformation of *Agrobacterium tumefaciens* with T-DNA vectors using high-voltage electroporation. Focus 15: 84–87.

Sive, H.L. and St John, T. 1988. A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. Nucl. Acids Res. 16: 10937.

Stanchev, B.S., Doughty, J., Scutt, C.P., Dickinson, H. and Croy R.R.D. 1996. Cloning of *PCP1*, a member of a family of pollen coat protein (*PCP*) genes from *Brassica oleracea* encoding novel cysteine-rich proteins involved in pollen-stigma interactions. Plant J. 10: 303–313.

Theerakulpisut, P., Xu, H., Singh, M.B., Pettitt, J.M. and Knox, R.B. 1991. Isolation and developmental expression of Bcp1, an anther-specific cDNA clone in *Brassica campestris*. Plant Cell 3: 1073–1084.

Twell, D. 1994. The diversity and regulation of gene expression in the pathway of male gametophyte development. In: R.J. Scott and A.D. Stead (Eds.), Molecular and Cellular Aspects of Plant Reproduction, Society for Experimental Biology, Cambridge University, Press, Cambridge, UK, pp. 83–135.

Ursin, V.M., Yamaguchi, J. and McCormick, S. 1989. Gametophytic and sporophytic expression of anther-specific genes in developing tomato anthers. Plant Cell 1: 727–736.

Wang, Z. and Brown, D.B. 1991. A gene expression screen. Proc. Natl. Acad. Sci. USA 88: 11505–11509.

Weterings, K., Reijnen, W., van Aarssen, R., Kortstee, A., Spijkers, J., van Herpen, M., Schrauwen, J. and Wullems, G. 1992. Characterization of a pollen-specific cDNA clone from *Nicotiana*

tabacum expressed during microgametogenesis and germination. Plant Mol. Biol. 18: 1101–1111.

Weterings, K., Schrauwen, J., Wullems, G. and Twell, D. 1995. Functional dissection of the promoter of the pollen-specific gene *NTP303* reveals a novel pollen-specific, and conserved *cis*-regulatory element. Plant J. 8: 55–63.

Woessner, J.P., Molendijk, A.J., van Egmond, P., Klis, F.M., Goodenough, U.W. and Haring, M.A. 1994. Domain conservation in several volvocalean cell wall proteins. Plant Mol. Biol. 26: 947–960.

Yu, L.-X., Nasrallah, J., Valenta, R. and Parthasarathy, M.V. 1998. Molecular cloning and mRNA localization of tomato pollen profilin. Plant Mol. Biol. 36: 699–707.